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Preferential Localization of Variant Nucleosomes Near the 5′-End of the Mouse Dihydrofolate Reductase Gene*

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We have probed the structure of nucleosomes within the 31-kilobase pair long, transcriptionally active gene for dihydrofolate reductase (DHFR) in mouse cells which contain multiple copies of the DHFR gene. We found that the distribution of electrophoretically variant nucleosomes within the DHFR gene is highly nonuniform: variant DHFR nucleosomes are abundant within and in the immediate vicinity of the ~200-base pair (bp) long first DHFR exon, and decrease by at least 10-fold within two nucleosomes upstream and downstream from this region. The nonuniformly distributed variant DHFR mononucleosomes are of two electrophoretically distinguishable discrete types. One corresponds to a mononucleosome containing a ~180-bp DNA fragment and possibly also histone H1 and high mobility group proteins. The other type of variant DHFR mononucleosome contains a ~146-bp DNA fragment, and its changes in relative content within the DHFR gene closely parallel those of the 180-bp variant mononucleosome. Several lines of evidence are consistent with the interpretation that the electrophoretically variant ~146-bp (core) mononucleosome species corresponds to diubiquitinated DHFR nucleosomes. We discuss possible causal relationships between the observed nonuniform distribution of variant nucleosomes within the DHFR gene and the DHFR gene transcription.

Spatial proximity between the two gyres of duplex DNA within the nucleosome (McGhee and Felsenfeld, 1980; Richmond *et al.*, 1984) suggests that negotiation of the nucleosome by a DNA template-dependent enzyme should be accompanied or preceded by a significant change in nucleosome structure. Indeed, actively transcribed eukaryotic genes, such as rRNA genes or heat shock genes in stressed cells, appear to be devoid of nucleosomal organization as probed using both electron microscopic and biochemical techniques (Miller and Beatty, 1969; Wu *et al.*, 1979; Levy and Noll, 1981; Labhart and Koller, 1982; Levinger and Varshavsky, 1982a; Davis *et al.*, 1983; Karpov *et al.*, 1984). Less actively transcribed genes, in which few RNA polymerase molecules traverse the gene simultaneously, have consistently been shown to possess nucleosomal organization; however, both the individual "active" nucleosomes and their higher order packing in the nucleus appear to differ from the corresponding structures in transcriptionally inert subsets of chromatin (reviewed by Cartwright *et al.*, 1982; see also Luchnik *et al.*, 1982; Prior *et al.*, 1983; Ryoji and Worcel, 1984). The reported differences include an increased overall sensitivity of transcribed chromosomal domains to nucleases, such as DNase I (Weintraub and Groudine, 1976; Garel and Axel, 1976), altered nucleosome repeat length (Smith *et al.*, 1983), undermethylation of transcribed DNA (reviewed by Doerfler, 1983) and also nucleosome-free regions, called "exposed" or "nuclease-hypersensitive" sites, present in particular near the 5′-ends of active or potentially active genes (Varshavsky *et al.*, 1978, 1979; Scott and Wigmore, 1978; Sundin and Varshavsky, 1979; Wu *et al.*, 1979; Wu, 1980; Stalder *et al.*, 1980; Jakobovitz *et al.*, 1980; Saragosti *et al.*, 1980; reviewed by Elgin, 1984). Nucleosomes from transcribed chromosomal regions appear to differ from bulk ("inactive") nucleosomes in their protein composition as well, in particular in the relative content of nonhistone proteins HMG14[1] and HMG17 (Weisbrod and Weintraub, 1981). Another protein apparently enriched in transcribed genes is ubiquitin H2A semihistone (uH2A) (Levinger and Varshavsky, 1982a; Varshavsky *et al.*, 1983), in which the carboxyl terminus of the small protein ubiquitin is joined via an isopeptide bond to the internal lysine 119 of histone H2A (Goldknopf *et al.*, 1975; Busch and Goldknopf, 1981; Wu *et al.*, 1981). uH2A substitutes for one or both of the nucleosomal H2A molecules in 10–20% of nucleosomes in higher eukaryotes (Levinger and Varshavsky, 1980; Busch and Goldknopf, 1981), and is present in cells in addition to a great variety of other, generally less abundant ubiquitin-protein conjugates, most of which are not DNA-bound (reviewed by Hershko, 1983; Ciechanover *et al.*, 1984a). Most of the latter ubiquitin-protein conjugates (but not necessarily uH2A) are intermediates in the ubiquitin-dependent proteolytic pathway that is responsible for the degradation of the bulk of short-lived intracellular proteins in eukaryotic cells (Hershko, 1983; Finley *et al.*, 1984; Ciechanover *et al.*, 1984b).

Using the technique of two-dimensional hybridization mapping of nucleosomes (Levinger *et al.*, 1981), it has been found

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[1] The abbreviations used are: HMG14 and HMG17, high mobility group proteins 14 and 17; DHFR, dihydrofolate reductase; PMSF, phenylmethylsulfonyl fluoride; NEM, *N*-ethylmaleimide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; rNTP, ribonucleoside triphosphate; SDS, sodium dodecyl sulfate; MN2, discrete mononucleosome intermediate containing ~160 to ~190-bp long DNA fragment, core histone octamer and one molecule of histone H1; MN1, core mononucleosome containing ~146-bp DNA fragment and core histone octamer but lacking histone H1, HMG proteins, and uH2A; $MN1_{uH2A}$, same but with one molecule of uH2A substituting for one molecule of histone H2A; $MN1_{(uH2A)_2}$, same but with two molecules of uH2A substituting for two molecules of H2A; uH2A, ubiquitin-H2A; kb, kilobase pairs; bp, base pairs; DNP, 2,4-dinitrophenol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DPT, *p*-diazophenylthioether.

that the relative content of apparently ubiquitinated nucleosomes is more than 10-fold higher within the transcribed *copia* and heat shock genes in nonstressed *Drosophila* cells than within transcriptionally inactive, satellite DNA-containing chromosomal regions in the same cells (Levinger and Varshavsky, 1982a, 1982b; Varshavsky *et al.*, 1983). In these studies, the distributions of variant nucleosomes were compared among different genes over extensive DNA lengths comparable with the sizes of the genes themselves. To determine whether the distribution of electrophoretically variant nucleosomes is modulated *within* a gene, we mapped variant nucleosomes within the 31 kilobase pair (kb) long gene for dihydrofolate reductase (DHFR) in mouse cells which contain multiple copies of the DHFR gene. The relative content of DHFR mRNA in such cells is increased over the parental cell's DHFR mRNA content approximately in proportion to the corresponding DHFR gene copy numbers (reviewed by Schimke *et al.*, 1981; Schimke, 1984), indicating that most if not all of the amplified DHFR genes are transcriptionally active. The increased hybridization signal due to the amplified state of the DHFR gene greatly facilitated high resolution nucleosome mapping.

MATERIALS AND METHODS

Cell Culture and in Vivo Labeling

Suspension cultures of mouse L5178Y-R cells (obtained from Dr. J. Bertino, Yale University) were maintained in tissue culture flasks (Corning) in Fischer's medium (GIBCO) containing 10% dialyzed horse serum (GIBCO), penicillin/streptomycin, and 0.5 mM methotrexate ((+)-amethopterin, Sigma). Monolayer cultures of 3T6-R500 cells (obtained from Dr. R. Kaufman) were grown in 15-cm tissue culture plates (Lux) in Dulbecco's modified Eagle's medium containing 10% dialyzed calf serum (GIBCO), penicillin/streptomycin, and 0.25 mM methotrexate.

Cells were labeled with [^{3}H]thymidine by adding [*methyl*-^{3}H]thymidine (20 Ci/mmol, New England Nuclear) to a final concentration of 1–5 μCi/ml for approximately 20 h. Labeling of proteins was carried out by adding L-[^{3}H]lysine (60 Ci/mmol, New England Nuclear) to a final concentration of 10 μCi/ml for approximately 24 h. In all of the labeling experiments cell cultures were in the exponential phase of growth.

Chromatin Preparation and Nuclease Digestion

Two procedures were used. All steps were carried out at 4 °C, unless otherwise stated. Treatment of suspended L5178Y-R cells was identical to that of 3T3-R500 monolayers except that cells in suspension were first pelleted at 500 × *g* for 5 min.

Protocol 1—Cell monolayers were rinsed twice with ice-cold 0.14 M NaCl, 5 mM Na/HEPES (pH 7.5), followed by the addition of 0.5% Nonidet P-40, 0.25 M sucrose, 5 mM Na/EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF; added shortly before use from a 0.1 M stock solution in ethanol), 10 mM *N*-ethylmaleimide (NEM; added shortly before use from a 0.1 M stock solution, pH 7.5), 10 mM Na/HEPES (pH 7.5). The lysate was scraped gently with a rubber policeman, suspended by pipetting with a 10-ml Falcon pipette, and thereafter centrifuged at 1000 × *g* for 5 min. The nuclear pellet was resuspended and washed twice in 0.14 M NaCl, 0.25 M sucrose, 1 mM Na/EDTA, 0.1 mM PMSF, 1 mM NEM, 10 mM Na/HEPES (pH 7.5). (In some of the experiments the 0.14 M NaCl wash was omitted; chromatin in such preparations was never exposed to an ionic strength significantly in excess of 10 mM; no significant change in DHFR-specific hybridization patterns was seen (data not shown).) The pellet was washed twice in 0.1 mM PMSF, 0.3 mM NEM, 1 mM Na/HEPES (pH 7.5) and gently resuspended to a final DNA concentration of approximately 1 mg/ml in 0.2 mM $CaCl_2$, 0.1 mM PMSF, 0.1 mM NEM, 1 mM Na/HEPES (pH 7.5). Staphylococcal nuclease (Sigma) was then added to a final concentration of 5 μg/ml, followed by digestion at 37 °C for 1–30 min. Digestion was stopped by the addition of 25 mM Na/EGTA, 50 mM Na/EDTA (pH 7.5) to final concentrations of 0.5 mM Na/EGTA and 1 mM Na/EDTA, respectively. After incubation at 0 °C for 30 min, the digest was centrifuged at 12,000 × *g* for 3 min. The supernatant was used either immediately or after storage at −70 °C in the presence of 10% glycerol.

Protocol 2—After rinsing the monolayers as described above, 9 ml of 0.25 M sucrose, 60 mM KCl, 15 mM NaCl, 0.5 mM spermidine, 14 mM 2-mercaptoethanol, 0.25 mM $MgCl_2$, 0.5 mM Na/EGTA, 0.5 mM PMSF, 5 mM NEM, 10 mM Na/HEPES (pH 7.5) were added per 3 × 10^8 cells, and the cells were gently scraped with a rubber policeman. Cells were then lysed with 25 strokes in a tight-fitting Dounce homogenizer. The lysate was layered on 2 volumes of 1.7 M sucrose in the same buffer and centrifuged in the SW-41 rotor (Beckman) at 25,000 rpm for 30 min at 4 °C. The nuclear pellet was washed and digested as described under Protocol 1. Although the two chromatin preparations differed in their nonhistone protein composition, no significant differences were seen in the corresponding DHFR-specific hybridization patterns (data not shown).

The presence of a thiol-blocking reagent (NEM) during chromatin isolation was necessary to prevent an otherwise nearly complete conversion of the chromosomal uH2A into H2A due to the activity of a PMSF-insensitive, NEM-sensitive isopeptidase in these preparations (see Barsoum *et al.*, 1982; Matsui *et al.*, 1982; Finley *et al.*, 1984).

Removal of Histone H1 from Chromatin by Exchange onto tRNA

A modification of the earlier technique (Ilyin *et al.*, 1971) was used. The nuclei were prepared as in Protocol 2. A suspension of single nuclei in 40 mM NaCl, 0.5 mM Na/EDTA, 0.1 mM PMSF, 0.5 mM NEM, 5 mM Na/HEPES (pH 7.5) was then prepared and mixed with purified total yeast tRNA (Sigma) to final tRNA and DNA concentrations of 3 mg/ml and ~0.1 mg/ml, respectively. The stock tRNA solution (100 mg/ml) was preliminarily dialyzed at 4 °C against 40 mM NaCl, 0.5 mM Na/EDTA, 5 mM Na/HEPES (pH 7.5). The tRNA-chromatin suspension was gently shaken for 2 h at 4 °C, followed by centrifugation at 1000 × *g* for 5 min and washing of the swelled [−H1]chromatin pellet twice with 0.1 mM PMSF, 0.1 mM NEM, 1 mM Na/HEPES (pH 7.5). The washed [−H1]chromatin (see Fig. 4) was resuspended and digested with staphylococcal nuclease as described above, except that the nuclease concentration was lowered to 2.5 μg/ml.

Two-dimensional Hybridization Mapping of DHFR Nucleosomes

Electrophoretic Fractionation of Nucleosomes—Low ionic strength 5% polyacrylamide gels (1.5 mm thick, 30 cm long) containing 0.5 mM Na/EGTA, 1 mM Na/EDTA, 10 mM Na/HEPES (pH 7.5) were run at 4 °C, followed by second dimension DNA runs in 9% polyacrylamide-SDS gels as described by Levinger *et al.* (1981), Levinger and Varshavsky (1982a), and Barsoum *et al.* (1982). To determine protein composition of nucleosome species separated in the first dimension, acetic acid-urea polyacrylamide gels were run in the second dimension as described by Levinger *et al.* (1981) except that the protamine concentration in the overlay was 10 mg/ml.

DNA Transfer and Hybridization—DNA was electrophoretically transferred from second dimension 9% polyacrylamide gels to DPT paper (prepared as described by Seed, 1982) after first denaturing DNA *in situ* by heating the gel to 100 °C (Levinger *et al.*, 1981). DPT papers (12.5 × 17.0 cm) were prehybridized for 15–24 h at 42 °C in 5 ml of 50% formamide, 5 × SSPE (1 × SSPE is 0.15 M NaCl, 1 mM Na/EDTA, 10 mM Na phosphate (pH 7.0)), 5 × Denhardt's solution (0.1% polyvinylpyrrolidone (M_r = 40,000; Sigma), 0.1% Ficoll (M_r = 40,000; Sigma), 0.1% bovine serum albumin)), and unlabeled denatured *Escherichia coli* DNA at 300 μg/ml (sonicated to a mean size of ~1 kb). Hybridization was carried out at 42 °C in the same medium with 150 μg/ml of the carrier *E. coli* DNA and 0.5–1 μg (~1 × 10^8 ^{32}P cpm/hybridization) of a specific hybridization probe labeled with ^{32}P by nick translation (Rigby *et al.*, 1977). After hybridization, DPT papers were washed 6 times in 500 ml of 0.2% SDS, 0.1 × SSPE at 52 °C and exposed at −70 °C to a Kodak XAR-5 film with a DuPont Lightning Plus intensifying screen. For rehybridization of the same DPT paper to a different probe, the paper was washed twice for 30 min each in 70% formamide, 0.2 × SSPE at 75 °C to remove the hybridized ^{32}P-DNA. After confirming (by a long autoradiographic exposure) the absence of ^{32}P on the paper, it was prehybridized, and then hybridized with a different probe as described above.

DHFR Hybridization Probes

The mouse DHFR cDNA and genomic DNA clones were gifts from Drs. R. Schimke and G. Crouse (Stanford University). Two genomic DHFR DNA clones were used: 1) pDR34, a 3.4-kb *Eco*RI DNA

fragment (cloned into pBR322) that spans the first two DHFR exons, most of the second DHFR intron and approximately 500 bp upstream of the first DHFR exon; 2) pDBg78, a 7.8-kb *Bgl*II DNA fragment (cloned into pMLB841) that spans the first two DHFR exons and also contains approximately 5.9 kb upstream of the first DHFR exon (Crouse *et al.*, 1982). All of the DHFR DNA probes used in this work, with the exception of probes IV, V, and X (see Fig. 1), were generated by digesting the above two cloned DNAs with restriction endonucleases (obtained from New England Biolabs and Bethesda Research Laboratories), fractionating the digests in agarose or polyacrylamide gels, and electroeluting the desired DNA fragments (see the legend to Fig. 1 for additional details). All of the DNA probes used were selected and tested in separate control experiments to behave as single-copy DNA sequences when Southern hybridized to DNA from parental cell lines not containing the amplified DHFR gene (data not shown). Several of the restriction sites shown in Fig. 1 were derived from maps by Crouse *et al.* (1982). We have mapped the rest of the sites using standard methods (Maniatis *et al.*, 1982).

RESULTS

Two-dimensional Hybridization Mapping of DHFR Nucleosomes: Preferential Localization of Variant Nucleosomes Near the 5′-End of the DHFR Gene—Two methotrexate-resistant mouse cell lines were used in this work. L5178Y-R is a lymphoblastoid line in which the DHFR gene is amplified approximately 300-fold relative to the parental cell line, L5178Y (Dolnick *et al.*, 1979; Barsoum *et al.*, 1982). In the fibroblast-like line 3T6-R500, the DHFR gene is amplified approximately 170-fold (Schimke *et al.*, 1981; Snapka and Varshavsky, 1983).

The ~31-kb long mouse DHFR gene (Nunberg *et al.*, 1980; Schimke *et al.*, 1981) contains six exons: the first five are each less than 200 bp long, and most of the sixth, 3′-terminal exon is noncoding (Fig. 1 and Crouse *et al.*, 1982). The initiator AUG codon for the DHFR enzyme in the first exon is preceeded by approximately 100 bp of noncoding DNA, placing a major DHFR mRNA cap site within a 4-fold repeated sequence at the 5′-end of the gene (Crouse *et al.*, 1982).

Isolated L5178Y-R chromatin (see "Materials and Methods") was digested with staphylococcal nuclease and soluble products were electrophoresed in a low ionic strength 5% polyacrylamide gel (Fig. 2*A*). It has been previously shown that this technique separates histone H1-containing from H1-lacking mononucleosomes (Varshavsky *et al.*, 1976; Todd and Garrard, 1977), and resolves the latter into core mononucleosomes (MN1) containing either 0, 1, or 2 molecules of uH2A/nucleosome, respectively (Levinger and Varshavsky, 1980, 1982a, 1982b; Swerdlow and Varshavsky, 1983). Second-dimension SDS-gel electrophoresis of DNA from mononucleosomes resolved in the first, deoxyribonucleoprotein (DNP) dimension yields a two-dimensional (DNP → DNA) pattern (Fig. 2*B*) in which positions of DNA spots are a function of nucleosome composition and conformation in the first (DNP) dimension. Thus, a transfer of two-dimensionally fractionated, denatured nucleosomal DNA fragments to a solid support, such as DPT paper (see "Materials and Methods"), and hybridization of the transferred DNA with specific DNA

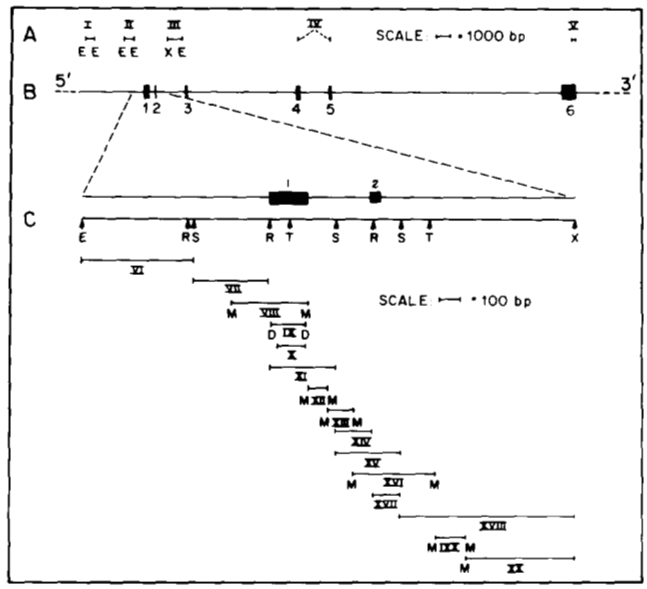

FIG. 1. **Genomic restriction map of the mouse dihydrofolate reductase gene, modified from Nunberg *et al.* (1980) and Crouse *et al.* (1982).** *A*, five DNA fragments (I–V) used as hybridization probes are shown above their respective positions in the DHFR gene. The *roman numerals* above each probe indicate the probe numbers used in the text. The *capital letters* beneath each fragment denote restriction endonucleases used for their excision (see below). Probes I and II were isolated from pDBg78 and probe III from pDR34 (see Crouse *et al.*, 1982 for description of the recombinant plasmids). Probe IV was isolated from a DHFR cDNA clone, pDHFR11 (Nunberg *et al.* (1980)); it thus lacks the intron between exons 4 and 5. The 5′-end of probe IV was produced by *Sac*I which cleaves 17 bp into exon 4, while the 3′-end was produced by *Hae*III which cleaves 20 bp into exon 5. Probe V contained the last 300 bp of exon 6; it was produced from pDHFR11 by digestion with *Pst*I and *Bgl*II. *B*, genomic map of the mouse DHFR gene. *Solid blocks* identify positions of the 6 DHFR exons (relative sizes of smaller exons are not precisely to scale). *C*, hybridization probes VI–XX are shown against an enlargement of the 2300-bp *Eco*RI-*Xba*I fragment at the 5′-end of the DHFR gene. All probes but X were isolated from pDR34 (Crouse *et al.*, 1982; see "Experimental Procedures"). Probe X was isolated from the cDNA clone pDHFR11 (Nunberg *et al.*, 1980) by digestion with *Pst*I and *Dde*I, which produced a 132-bp DNA probe that lacks 4 bp and approximately 130 bp at the 3′- and 5′-ends, respectively, of exon 1. The *capital letters* beneath each fragment denote restriction sites (*E* = *Eco*RI; *X* = *Xba*I; *R* = *Rsa*I; *S* = *Sau*3a; *T* = *Taq*I; *M* = *Msp*I; *D* = *Dde*I). Where no enzyme is indicated, the cleavage is due to a restriction endonuclease whose sites are shown in map *C*. Initial genomic and cDNA DHFR clones used in this work were generously provided by Drs. R. Schimke and G. Crouse.

FIG. 2. **Two-dimensional hybridization mapping of DHFR-specific nucleosomes from L5178Y-R cells using DHFR exon probes.** Nucleosomes in early (2.5% acid-soluble; column I) and late (6% acid-soluble; column II) staphylococcal nuclease digests of L5178Y-R chromatin were resolved by two-dimensional (DNP → DNA) electrophoresis, followed by hybridization to probes derived from the cloned DHFR cDNA (see "Materials and Methods" and Fig. 1). *A*, total (ethidium-stained) first-dimension (DNP) pattern. Mono- and dinucleosomal region of the gel is shown. *B*, total (ethidium-stained) two-dimensional (DNP → DNA) pattern. *C*, DNA in *B* was transferred to DPT paper and hybridized with the ^{32}P-labeled, full-length DHFR cDNA clone (pDHFR11; see Fig. 1). *D*, same DPT paper was rehybridized with the ^{32}P-labeled probe X specific for exon 1 (see Fig. 1). Note a striking enhancement of hybridization signal in the $MN1_{(uH2A)_2}$ DNA spot (*arrowhead, panel DII*), and also in the two discrete DNA spots behind MN2 (*arrows, panels DI* and *DII*). *E*, same DPT paper was rehybridized with the ^{32}P-labeled probe V (last 300 bp of the DHFR gene, exon 6; see Fig. 1). Terminology: *DN*, dinucleosomes; *MN2*, discrete mononucleosome intermediate containing ~160 to ~190-bp long DNA fragment, core histone octamer and one molecule of histone H1; *MN1*, core mononucleosome containing ~146-bp DNA fragment and core histone octamer but lacking histone H1, HMG proteins, and uH2A; $MN1_{uH2A}$, same but with one molecule of uH2A substituting for one molecule of histone H2A; $MN1_{(uH2A)_2}$, same but with two molecules of uH2A substituting for two molecules of H2A. Some of the differences between hybridization patterns are indicated by *arrows* and an *arrowhead*.

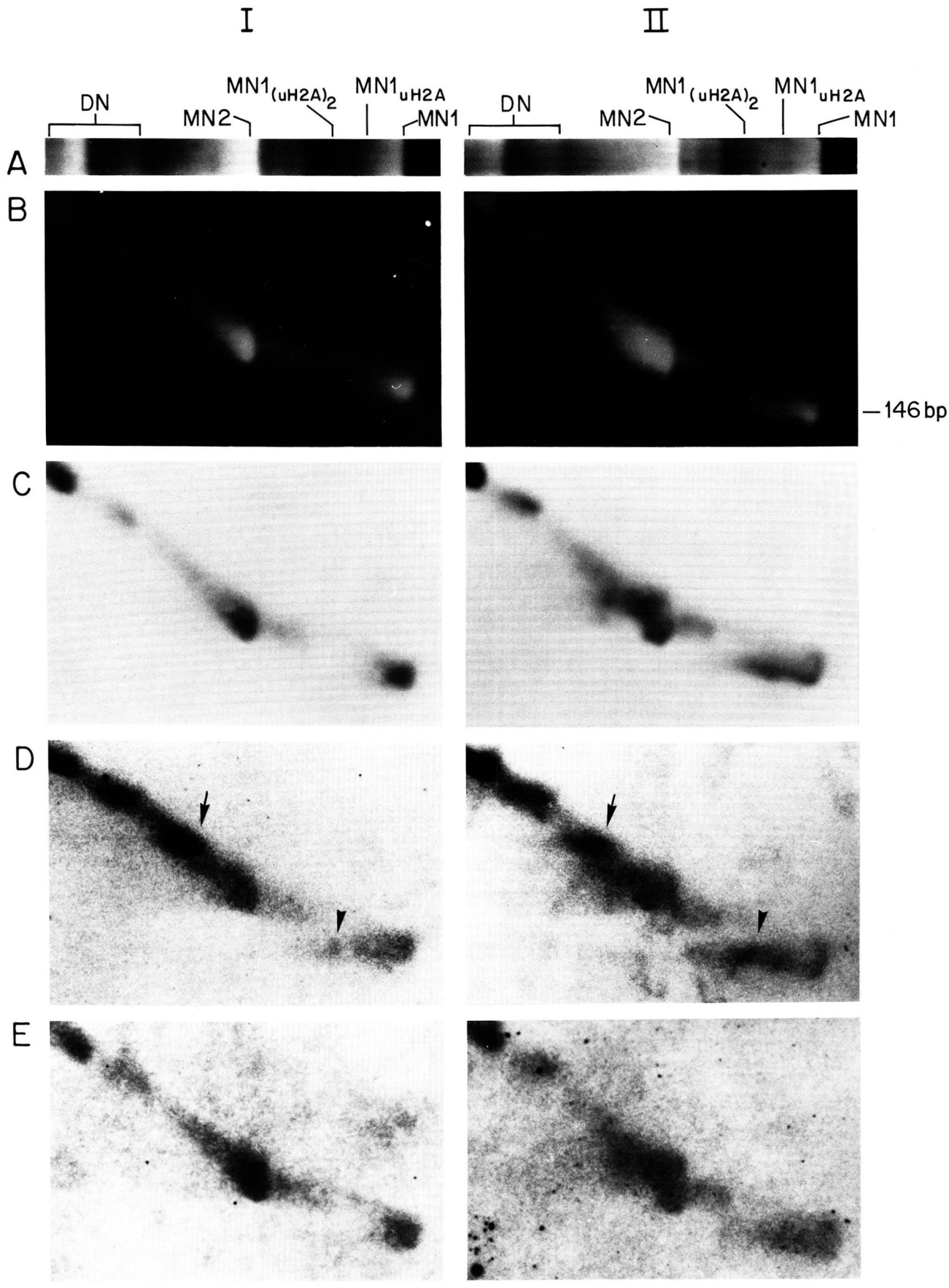

FIG. 2

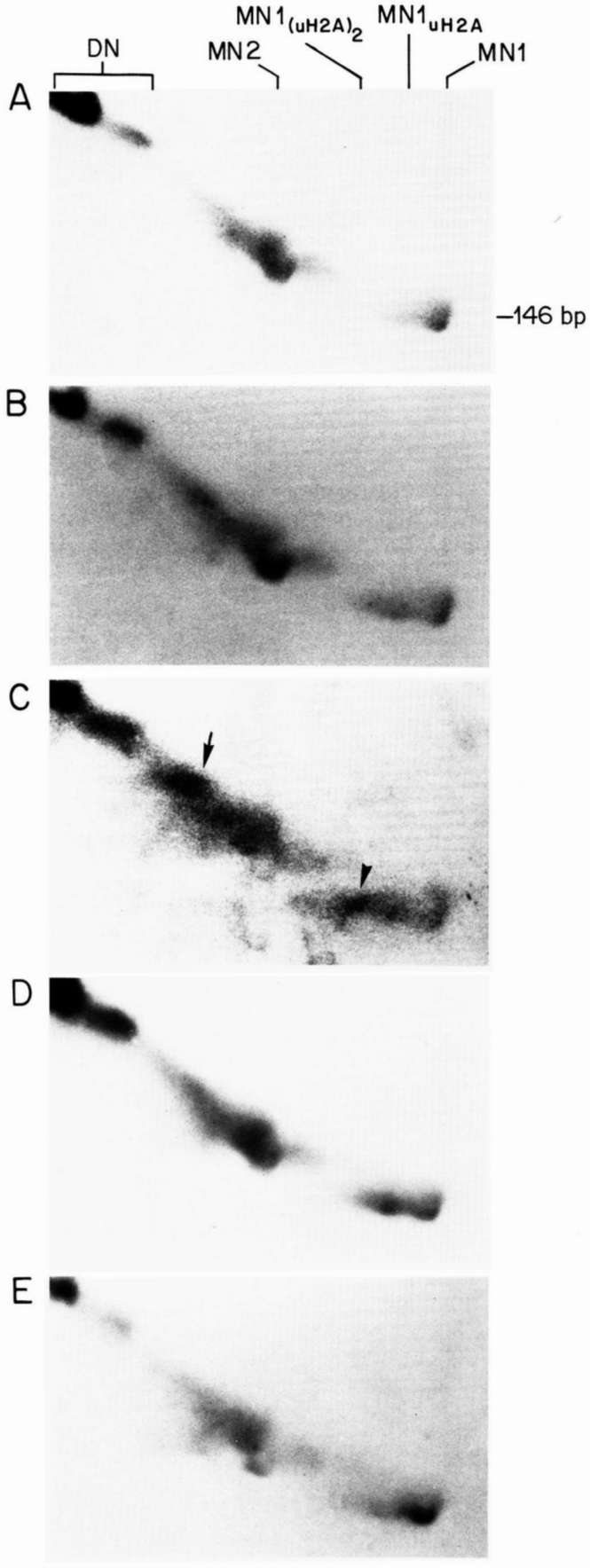

FIG. 3. **Two-dimensional hybridization mapping of nucleosomes from L5178Y-R cells with probes specific for the 5′-region of the DHFR gene.** The same DPT paper used for hybridization mapping of nucleosomes in Fig. 2 (Panels *BII–EII*) was rehybridized successively with other DNA probes (ethidium-stained total DNA pattern is shown in Fig. 2*AII*). See Fig. 1 for the map positions of specific probes used (I–III, X, and XVI). *A*, probe I; *B*, probe II; *C*, probe X (same as in Fig. 2*IIC*); *D*, probe XVI; *E*, probe III. Some of the differences between hybridization patterns are indicated by *arrows* and *arrowheads*. Note also the internal heterogeneity of the MN1 DNA spot: probes II and X preferentially hybridize to the most rapidly migrating MN1 DNP species within the MN1 DNA spot (*panels B* and *C*), whereas the probes III and XVI hybridize uniformly along the DNP dimension of the MN1 DNA spot, but display a strong preference for the smaller DNA fragments within the same spot (*panels D* and *E*).

probes allows one to study the protein composition and conformation of subsets of nucleosomes containing DNA sequences hybridizing to the probe used (Levinger *et al.*, 1981).

When DNA in the two-dimensional (DNP → DNA) pattern of total mononucleosomes from L5178Y-R cells (Fig. 2, *BI* and *BII*) is hybridized to a cloned, ^{32}P-labeled DHFR cDNA (pDHFR11), no large differences are seen between the bulk and hybridization patterns (Fig. 2, *BI*, *CI* and *BII*, *CII*). One reproducibly detectable difference is an approximately 2-fold increase in the relative content of apparently monoubiquitinated core mononucleosomes ($MN1_{uH2A}$) in the hybridization pattern (Fig. 2*CII*) as compared with the corresponding bulk pattern (Fig. 2*BII*).

Approximately 60% of the ~1600-bp DHFR cDNA probe used in Fig. 2*C* is occupied by the 1040-bp 3′-terminal sixth exon, located more than 30 kb from the 5′-end of the DHFR gene (Crouse *et al.*, 1982). The 5′-terminal (first) exon constitutes less than 15% of this exon-specific probe (Fig. 1). To see whether smaller subsets of the DHFR gene produce different hybridization patterns, we rehybridized *the same DPT paper* with the first exon-specific, 132-bp fragment (probe X in Fig. 1), and with other DHFR DNA probes (gifts from Drs. R. Schimke and G. Crouse), together spanning the length of the DHFR gene (see Fig. 1 and "Materials and Methods").

To simplify description of the results to follow, we shall assume that DHFR-specific hybridization patterns which coincide with the *bulk* $MN1_{uH2A}$ and $MN1_{(uH2A)_2}$ DNA spots signify the presence of monoubiquitinated and diubiquitinated nucleosomes, respectively, in the corresponding regions of the DHFR gene (see Varshavsky *et al.*, 1983; Wu *et al.*, 1983, for discussion and references). It should be emphasized, however, that although this is the simplest interpretation which is also consistent with other lines of evidence (see below), it does not directly follow from the data.

Hybridization patterns observed with the 5′-end specific DHFR probes were, in the following several respects, strongly different from the corresponding patterns observed with either the complete DHFR cDNA or with 3′-end specific DHFR probes:

(i) A discrete hybridization spot (indicated by *arrowhead*) is present at the position expected for the diubiquitinated core mononucleosome ($MN1_{(uH2A)_2}$) (Levinger *et al.*, 1981; Levinger and Varshavsky, 1982a; Swerdlow and Varshavsky, 1983) when probe X is used (Figs. 2, *DI* and *DII* and 3*C*) but is not seen in the total pattern or when a complete DHFR cDNA probe is used (Fig. 2, *B* and *C*). The 132-bp probe X (Fig. 1) is entirely contained within the ~200 bp first DHFR exon, and lacks the 5′ most part of this exon. The at least 10-fold enhancement of the $MN1_{(uH2A)_2}$ mononucleosome species is seen with probe X in both relatively light (Fig. 2*DI*) and extensive (Fig. 2*DII*) staphylococcal nuclease digests. The $MN1_{(uH2A)_2}$ mononucleosome is an extremely minor species in *bulk* mononucleosomal DNP → DNA patterns, being approximately 10- and 50-fold less abundant than its monoubiquitinated ($MN1_{uH2A}$) and nonubiquitinated (MN1) counterparts, respectively (Fig. 2*B*; see also Levinger *et al.*, 1981;

Varshavsky *et al.*, 1983). In striking contrast, the $MN1_{(uH2A)_2}$ nucleosome seen with probe X within the first DHFR exon is about as abundant in this region of the DHFR gene as are the monoubiquitinated ($MN1_{uH2A}$) and nonubiquitinated (MN1) nucleosomes (Fig. 2*D*). In control experiments, the pattern of enhanced $MN1_{(uH2A)_2}$ hybridization with probe X was seen also in a preparation of 11 S mononucleosomes purified first by sedimentation in a sucrose gradient (data not shown), thus demonstrating that slower migration of the variant MN1 mononucleosome species in Fig. 2*D* is not due to an unfolded state of the DNA, since unfolded particles of comparable total mass would sediment at 5–6 S (see Prior *et al.*, 1983).

(ii) Another feature of the probe X-specific hybridization pattern is the intense hybridization spot (indicated by *arrow*) on the diagonal behind the MN2 DNA species (Fig. 2, *DI* and *DII*). This spot, whose relative intensity exceeds that of the MN2 spot in the same hybridization pattern (Fig. 2*D*) is practically undetectable in both the bulk pattern (Fig. 2*B*) and the full length cDNA-specific hybridization pattern (Fig. 2*C*). The electrophoretic mobility of the corresponding DNP particle is consistent with it being an HMG-containing nucleosome (see Levinger *et al.*, 1981; Barsoum *et al.*, 1982). This assignment remains tentative, however, for reasons alluded to above.

(iii) The probe X-specific hybridization patterns (Figs. 2*D* and 3*C* and data not shown) required approximately 4-fold longer autoradiographic exposures than the patterns specific for the DHFR probes *outside* the 5′-end of the DHFR gene to obtain comparable final levels of signal (Figs. 2 and 3, and data not shown). This difference was highly reproducible, was not found with other small probes, and was not due to lower specific radioactivities of the 5′-end-specific DHFR probes (IX–XI; see Fig. 1). The lower yield of the 5′-end proximal DHFR mononucleosomes as compared with the 5′-end distal DHFR mononucleosomes in both light and extensive staphylococcal nuclease digests indicates that in a significant proportion of the DHFR genes their 5′-end proximal regions are not processed by staphylococcal nuclease into structures identifiable as nucleosomes by electrophoresis and sedimentation (see "Discussion").

(iv) When DNA in the DNP → DNA mononucleosomal pattern of Fig. 2*IIB* was rehybridized with the ~900-bp long probe I centered approximately 4 kb upstream from the first DHFR exon (Fig. 1), the resulting hybridization pattern (Fig. 3*A*) was not significantly different from either the bulk pattern (Fig. 2*IIB*) or the pattern from the 3′-end of the DHFR gene (Fig. 2*IIE*), in great contrast with the first exon-specific pattern (Figs. 2*IID* and 3*C*).

(v) Rehybridization of the same DPT paper with the ~700-bp long probe II located ~600 bp upstream from the first DHFR exon (Fig. 1), produced a pattern (Fig. 3*B*) "intermediate" between those for the far upstream probe I (Fig. 3*A*) and the first exon-specific probe X (Fig. 3*C*): while no $MN1_{2(uH2A)}$ DNA spots are detected with probe II (Fig. 3*B*; compare with probe X, Fig. 3*C*), the relative content of monoubiquitinated ($MN1_{uH2A}$) mononucleosomes is clearly higher with probe II (located ~600 bp upstream from the first DHFR exon) than with probe I (located ~4 kb upstream from the first exon).

(vi) As noted above, the further downstream, first exon-specific probe X reveals in particular an at least 10-fold increase in the content of the specific MN1 mononucleosome subspecies interpreted as the doubly ubiquitinated DHFR mononucleosome, $MN1_{(uH2A)_2}$; however, the relative abundance of the $MN1_{(uH2A)_2}$ mononucleosome falls to a practically undetectable (bulk) level *less than two nucleosomes further downstream*. This is demonstrated in Fig. 3*D*, which shows the hybridization pattern of the 383-bp long probe XVI (Fig. 1), whose 5′-end is approximately 220 bp downstream from the first exon probe X. This decrease in relative intensity of the $MN1_{(uH2A)_2}$ DNA spot within two nucleosome lengths downstream from the nucleosome corresponding to the first DHFR exon is paralleled by an equally strong decrease in a specific mononucleosome species that migrates behind the MN2 mononucleosome. This variant nucleosome, possibly an HMG-containing one (indicated by *arrow* in Figs. 2 and 3; see item ii above), is prominent within the first DHFR exon, and is virtually absent both upstream and downstream from this region.

(vii) Probe III, located within the second DHFR intron, 650 bp downstream from probe XVI (Fig. 1), produces a pattern in which the $MN1_{(uH2A)_2}$ component is absent, and the content of $MN1_{uH2A}$ is further decreased (Fig. 3*E*; compare with Fig. 3, *C* and *D*), making the probe III-specific pattern (Fig. 3*E*) practically indistinguishable from both the bulk and the 3′-end specific patterns (Figs. 2 and 3, and data not shown).

(viii) Another feature of the MN1 hybridization patterns in Fig. 4 is that probes corresponding to different regions of the DHFR gene yield *nonubiquitinated* core mononucleosome patterns which comprise *specific subsets* of an apparently discrete bulk MN1 DNA spot. For instance, probes II and X preferentially hybridize to the most rapidly migrating MN1 DNP species (leading edge of the MN1 DNA spot; Fig. 3, *B* and *C*), whereas probes III and XVI hybridize uniformly along the DNP dimension of the MN1 DNA spot, but display a strong preference for the smaller DNA fragments within the same spot (Fig. 3, *D* and *E*; compare with Fig. 3, *B* and *C*). Whatever the structural basis of this previously unreported microheterogeneity, it is not likely to be due to variations in DNA sequence alone, as neighboring DHFR nucleosomes tend to share the MN1 spot microheterogeneity patterns (Fig. 3).

In our earlier work (Barsoum *et al.*, 1982), the presence of a highly active, PMSF-resistant isopeptidase in L5178Y-R chromatin preparations resulted in the nearly complete conversion of uH2A into H2A histone, unless a thiol-blocking reagent, such as NEM, was present during chromatin isolation[2] (see also Matsui *et al.*, 1982; Finley *et al.*, 1984). Inhibition of isopeptidase with NEM in the present work yielded chromatin whose content of uH2A was close to that of other mammalian chromatin preparations (data not shown; see also Levinger *et al.*, 1981; Busch and Goldknopf, 1981). Experiments of the type shown in Figs. 2 and 3, when carried out in the absence of NEM, showed not only the expected disappearance of uH2A semihistone and of the corresponding bulk $MN1_{uH2A}$ nucleosomal DNA spots (see Barsoum *et al.*, 1982), but also the disappearance of the putative doubly ubiquitinated $MN1_{(uH2A)_2}$ mononucleosome in hybridization patterns with the DHFR first exon-specific DNA probes (Figs. 2 and 3, and data not shown). These data are consistent with the interpretation that most of the slower migrating, DHFR gene-specific MN1 mononucleosomes correspond to ubiquitinated nucleosome species.

Removal of Histone H1 from Chromatin Does Not Change the Distribution of Variant Nucleosomes within the DHFR Gene—We asked whether the patterns of variant DHFR nucleosomes (Figs. 2 and 3) depended on the presence of less tightly DNA-bound proteins, such as histone H1, in chromatin before nuclease digestion. One mild procedure for selective removal of histone H1 from chromatin is based on the ability of H1 but not of core histones to exchange rapidly between H1 binding sites in chromatin and added transfer RNA (Ilyin *et al.*, 1971; Varshavsky and Ilyin, 1974).

[2] D. Finley and J. Barsoum, unpublished data.

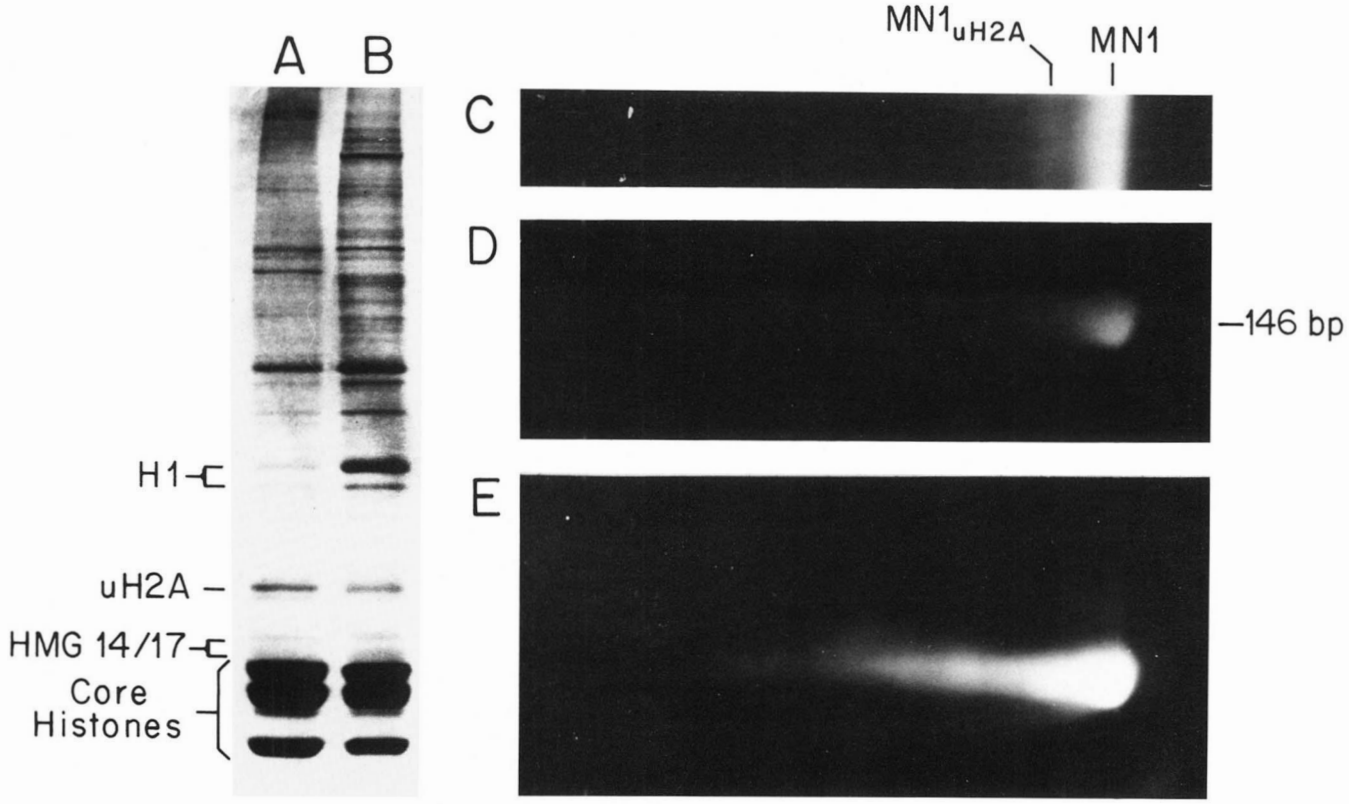

FIG. 4. **Removal of histone H1 from 3T6-R500 chromatin by exchange onto tRNA.** More than 90% of histone H1 and a subset of nonhistone proteins were removed from 3T3-R500 chromatin by tRNA-mediated exchange (see "Materials and Methods") followed by staphylococcal nuclease digestion and two-dimensional fractionation of nucleosomes. *A*, unidimensional, Coomassie-stained SDS-gel electrophoretic pattern of proteins from chromatin after the tRNA-mediated H1 removal. *B*, same but the initial, untreated chromatin. *C*, total (ethidium-stained) first-dimension (DNP) pattern of nucleosomes from the [−H1]chromatin. *D*, total (ethidium-stained) two-dimensional (DNP → DNA) pattern. *E*, Same as in *D* but staining with a higher concentration of ethidium bromide.

We used 3T6-R500 cells (see above) for preparation of [−H1]chromatin (Fig. 4). These cells were also tested in experiments identical to the ones with L5178Y-R cells and H1-containing chromatin; the results were closely similar to those in Figs. 2 and 3 (data not shown). Treatment of the 3T6-R500 chromatin with tRNA removed more than 90% of the original H1, as well as a number of specific nonhistone protein species (Fig. 4, *A* and *B*); however, most of the chromatin-bound HMG14 and HMG17 proteins are not removed by this procedure (data not shown). Furthermore, the [−H1]chromatin thus obtained retains its original nucleosome repeat length,[3] in contrast to [−H1]chromatins obtained by salt extraction methods (Varshavsky and Ilyin, 1974).

Staphylococcal nuclease digestion of [−H1]chromatin produces mononucleosomes as major metastable intermediates even at early stages of digestion; such mononucleosomes consist almost exclusively of ~146 bp MN1 species with virtually no MN2 nucleosomes (Fig. 4*C*). Two-dimensional (DNP → DNA) fractionation of mononucleosomes from [−H1]chromatin yields the pattern shown in Figs. 4, *D* and *E*, and 5*A*, in which the major DNA spot of nonubiquitinated MN1 mononucleosomes is followed by a minor spot of $MN1_{uH2A}$; in addition, a minor streak of 146-bp mononucleosomes that migrate slower than both $MN1_{uH2A}$ and $MN1_{(uH2A)_2}$ but faster than MN2 in the first (DNP) dimension, is seen upon overstaining of the bulk pattern (Fig. 4*E*). These minor ~146-bp DNA species may correspond to MN1 mononucleosomes containing HMG14 and HMG17 proteins (see Sandeen *et al.*, 1980; Mardian *et al.*, 1980; Levinger *et al.*, 1981; Swerdlow and Varshavsky, 1983; Stein and Townsend, 1983).

When DNA in the DNP → DNA mononucleosomal pattern of Fig. 5*A* was hybridized to probe I which is centered approximately 4 kb upstream from the first DHFR exon (Fig. 1), the resulting hybridization pattern was not significantly different from the bulk one at both relatively low and high levels of autoradiographic exposure (Fig. 5, *B* and *C*). A similar result was obtained with probe II located ~600 bp upstream from the first DHFR exon (Figs. 1 and 5*D*). Rehybridization of the same DPT paper with the 365-bp long probe VII located immediately upstream of the first DHFR exon (Fig. 1), produced a pattern (Fig. 5*E*) similar although not identical to those obtained with probes I and II (Fig. 5, *B–D*). However, when the first exon-specific, 159-bp long probe IX which is located immediately downstream from probe VII was used (Fig. 1), the pattern obtained (Fig. 5*F*) was strikingly different from those obtained with probes I, II, and VII (Fig. 5, *B–E*). First, in addition to a strong relative enhancement of the $MN1_{uH2A}$ DNA spot, probe IX revealed the $MN1_{(uH2A)_2}$ spot that was undetectable with even the "immediately upstream" probe VII (Fig. 5*F*; compare with Fig. 5*E*). Second, probe IX hybridized to an isolated horizontal streak of ~146-bp DNA fragments (Fig. 5*F*, *arrow*), corresponding to specific variant mononucleosomes; their first-dimension (DNP) mobility coincides with that of bulk MN1 nucleosomes containing two HMG14/HMG17 molecules/particle and no H1 histone

[3] A. Varshavsky, unpublished data.

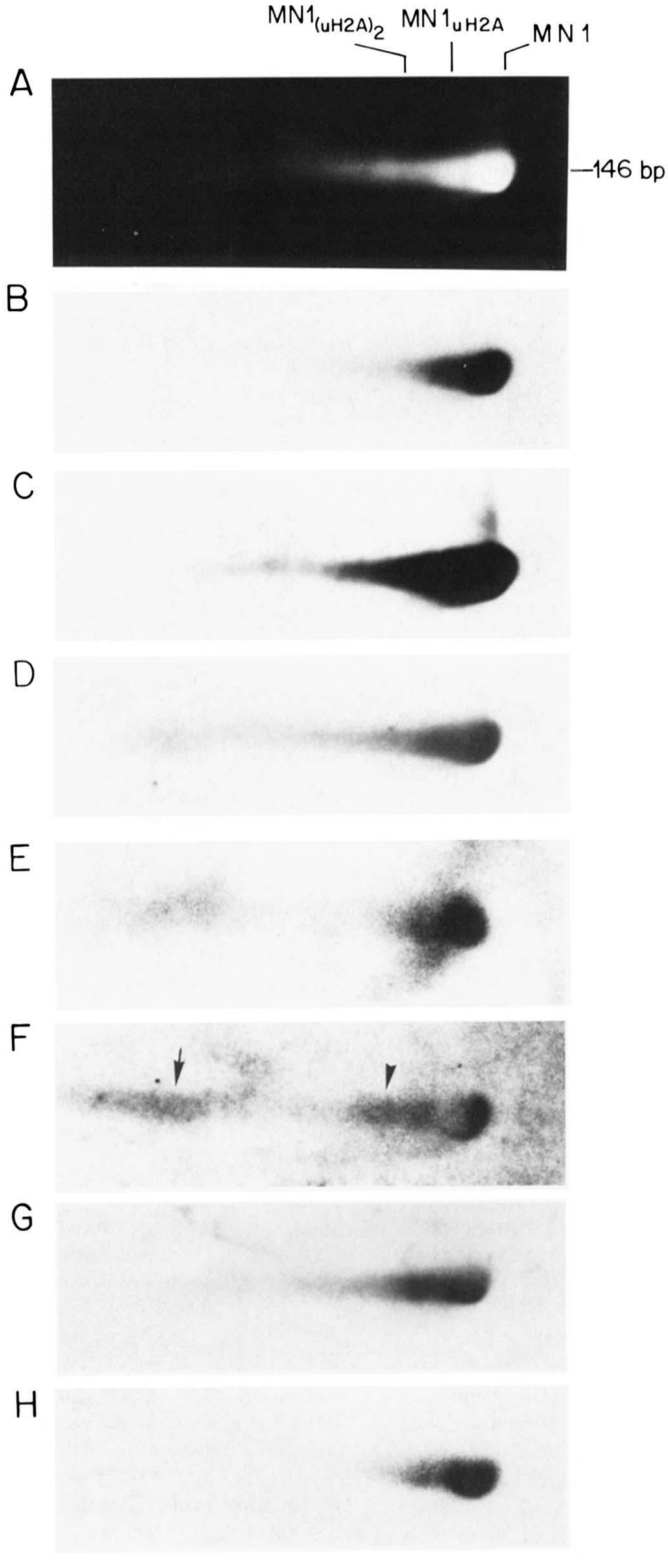

FIG. 5. **Two-dimensional hybridization mapping of DHFR-specific nucleosomes from [−H1]chromatin.** *A*, total (ethidium-stained) two-dimensional (DNP → DNA) pattern (same as in *E*). *B*, DNA in *A* was transferred to DPT paper and hybridized with the ^{32}P-labeled probe I (see Fig. 1 for the map positions of specific probes used). *C*, same as in *B* but a longer autoradiographic exposure. *D–H*, same DPT paper was rehybridized successively with probes II, VII, IX, XIV, and IXX, respectively. Some of the differences between the hybridization patterns are indicated by *arrows* and *arrowheads*.

(Swerdlow and Varshavsky, 1983). These nucleosome species (Fig. 5*F*, *arrow*) are completely undetectable with probes I and II (Fig. 5, *C* and *D*) and are barely detectable with the immediately upstream probe VII (Fig. 5*E*). Third, in contrast to probes I, II, and VII, probe IX (Fig. 1) hybridizes to a *subset* of nonubiquitinated MN1 mononucleosomes, forming a crescent-shape hybridization spot (Fig. 5*F*; see also item viii above and Fig. 3*C*).

As was already seen with DHFR nucleosomes from H1-containing chromatin (Figs. 2 and 3), the relative content of variant DHFR MN1 mononucleosomes from [−H1]chromatin decreases greatly just one nucleosome length downstream from the first DHFR exon (Fig. 5*G*) and decreases further to approximately the bulk content of variant nucleosomes less than 600 bp downstream from the end of the first DHFR exon (Fig. 5*H*).

Thus the striking enrichment in variant nucleosomes in the immediate vicinity of the first DHFR exon (Fig. 1) is observed with nucleosomes from both histone H1-containing and H1-lacking chromatin. Furthermore, as mentioned above, all of the variant DHFR nucleosome species (Figs. 3 and 5, *arrows* and *arrowheads*) co-sediment with bulk 11 S mononucleosomes in a sucrose gradient. Taken together, these results indicate that conformational differences alone are not sufficient to account for the electrophoretic behavior of variant DHFR nucleosomes, and that compositional differences must be involved as well.

DISCUSSION

One interpretation of the above results which appears to be both mechanistically plausible and consistent with the available evidence is that the steady-state content of variant nucleosomes within a given chromosomal region is directly dependent on the mean temporal frequency of RNA polymerase passages through that region. Implicit in this hypothesis is the assumption that the content and distribution of variant nucleosomes within a gene at any given moment reflects a dynamic equilibrium between, on the one hand, reactions of nucleosome modification that occur prior to, concurrently with or, less likely, after the passage of transcribing RNA polymerase through a gene region, and, on the other hand, demodification or replacement of variant nucleosomes in the same region. To account (in the framework of the above model) for the greatly increased content of variant nucleosomes in the first ~500 bp of the ~31-kb long DHFR gene one could suggest that the relative content of transcribing RNA polymerases within the first ~500 bp of the DHFR gene is higher than in the rest of the DHFR gene. The results of our preliminary *in vitro* transcription experiments with Sarkosyl nuclear lysates of 3T6-R500 cells do indeed suggest that the relative content of transcribing RNA polymerases is higher at the beginning of the DHFR gene and decreases strongly less than 1 kb downstream.[4] This apparent transcriptional attenuation (Kolter and Yanofsky, 1982; Skolnik-David and Aloni, 1983) of the DHFR gene[4] is consistent with the known cell cycle dependence of DHFR gene expression (Mariani *et al.*, 1981; Leys and Kellems, 1981; Kaufman and Sharp, 1983; Santiago *et al.*, 1984) which appears to be due to both transcriptional and post-transcriptional controls (Farnham and Schimke, 1985). More detailed analyses of DHFR transcriptional patterns will be required to directly test the above hypothesis that the observed enrichment is variant nucleosomes near the 5′-end of the DHFR gene (see "Results") is causally related to the distribution of transcribing RNA polymerases within the DHFR gene.

[4] J. Barsoum and A. Varshavsky, unpublished data.

Mechanistic aspects of nucleosome modification processes are largely unknown. For instance, it is unclear whether ubiquitinated nucleosomes are eventually converted back into bulk nucleosomes directly by deubiquitination, or whether more complex pathways are involved (see below). It also remains to be established whether the major role of transcription-related nucleosome modifications outside of promoter regions is to facilitate negotiation of nucleosomes by RNA polymerase, or whether additional essential functions, such as a change in susceptibility of a chromosomal region containing variant nucleosomes to the action of DNA topoisomerases (Luchnik *et al.*, 1982; Ryoji and Worcel, 1984), are involved as well. Finally, it is not clear whether nucleosome modification patterns are established within a previously inactive gene via a cis-acting process directly dependent on the first passage of RNA polymerase through the gene, or whether transcription-independent mechanisms are also involved.

Possible Significance of Nucleosomal Ubiquitin-Histone Conjugates—Ubiquitin has recently been shown by both biochemical and genetic approaches to serve as an essential cofactor in ATP-dependent degradation of the bulk of short-lived intracellular proteins in eukaryotic cells (Hershko, 1983; Finley *et al.*, 1984; Ciechanover *et al.*, 1984b, and references therein). Nonetheless, as the bulk of DNA-bound histones turns over slowly, the significance of uH2A, uH2B, and other ubiquitin-histone conjugates may be unrelated to ubiquitin-dependent proteolysis. Among the possible nonproteolytic roles of nucleosome ubiquitination are transient perturbation (unfolding) of chromatin structures possibly including the nucleosome itself, and marking specific regions of chromosomal fibers for binding of unidentified ligands involved in chromosomal processes such as transcription, replication, or repair.

Although none of the available evidence directly supports the hypothesis of chromosomal locus-specific, ubiquitin-dependent proteolysis of histones as a mechanism of chromatin modification (Levinger and Varshavsky, 1982a; Varshavsky *et al.*, 1983), we are aware of no data that directly contradict it. One specific hypothesis is that the extensive ubiquitination of nucleosomes near the 5′-end of the DHFR gene (see "Results") is due to their location proximal to a nucleosome-free exposed region, such as found near 5′-ends of other active genes (see Introduction). Such nucleosome-free structures are present only conditionally in chromatin (Varshavsky *et al.*, 1979; Groudine and Weintraub, 1982), and apparently replace pre-existing nucleosome structures. To find nucleosome ubiquitination directed preferentially at or near an exposed region would suggest that nucleosome removal may involve ubiquitin-dependent proteolysis. To address this problem will require some means to specifically perturb ubiquitination and other nucleosome modifications *in vivo*. The recent identification of the mouse cell cycle mutant ts85 as a ubiquitin pathway (ubiquitin-activating enzyme) mutant (Finley *et al.*, 1984; Ciechanover *et al.*, 1984b) and the more recent isolation of ubiquitin pathway mutants in yeast[5] may now provide definitive tools to address the functions of ubiquitin-histone conjugates in chromosomal processes.

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[5] D. Finley and A. Varshavsky, unpublished data.

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